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Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates

(chemoprotection/quinone reductase/enzyme induction/dimethylbenzanthracene/rat mammary tumors)

YUESHENG ZHANG*, THOMAS W. KENSLE†, CHEON-GYU CHO‡, GARY H. POSNER‡, AND PAUL TALALAY*§

*Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; †Department of Environmental Health Sciences, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD 21205; and ‡Department of Chemistry, The Johns Hopkins University School of Arts and Sciences, Baltimore, MD 21218

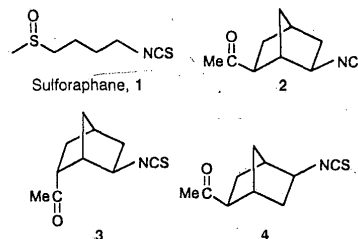
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ABSTRACT Sulforaphane [1-isothiocyanato-4-(methylsulfinyl)butane] was recently isolated from one variety of broccoli as the major and very potent inducer of phase 2 detoxication enzymes in murine hepatoma cells in culture. Since phase 2 enzyme induction is often associated with reduced susceptibility of animals and their cells to the toxic and neoplastic effects of carcinogens and other electrophiles, it was important to establish whether sulforaphane could block chemical carcinogenesis. In this paper we report that sulforaphane and three synthetic analogues, designed as potent phase 2 enzyme inducers, block the formation of mammary tumors in Sprague-Dawley rats treated with single doses of 9,10-dimethyl-1,2-benzanthracene. The analogues are *exo*-2-acetyl-*exo*-6-isothiocyanatonorbornane, *endo*-2-acetyl-*exo*-6-isothiocyanatonorbornane, and *exo*-2-acetyl-*exo*-5-isothiocyanatonorbornane. When sulforaphane and *exo*-2-acetyl-*exo*-6-isothiocyanatonorbornane were administered by gavage (75 or 150 μ mol per day for 5 days) around the time of exposure to the carcinogen, the incidence, multiplicity, and weight of mammary tumors were significantly reduced, and their development was delayed. The analogues *endo*-2-acetyl-*exo*-6-isothiocyanatonorbornane and *exo*-2-acetyl-*exo*-5-isothiocyanatonorbornane were less potent protectors. Thus, a class of functionalized isothiocyanates with anticarcinogenic properties has been identified. These results validate the thesis that inducers of phase 2 enzymes in cultured cells are likely to protect against carcinogenesis.

Enzymes that metabolize xenobiotics play a major role in regulating the toxic, mutagenic, and neoplastic effects of chemical carcinogens. Much evidence indicates that the activities of phase 2 detoxication enzymes (e.g., glutathione transferases, NAD(P)H:quinone reductase, UDP-glucuronosyltransferases, and epoxide hydrolase) in particular can modulate the response of animals and their cells to carcinogen exposure. Induction of these enzymes by a wide variety of chemicals (including components of the diet) results in protection against toxicity and neoplasia (1). To identify such protective inducers and to measure their potencies, a simple cell culture system has been developed in our laboratory (2, 3). This system depends on determining the specific activities of quinone reductase in murine hepatoma cells grown in 96-well microtiter plates and exposed to a range of concentrations of the inducers. Such measurements not only have reliably predicted the ability of compounds to induce phase 2 enzymes in rodent tissues *in vivo* but also have identified several chemoprotectors against carcinogenesis. By use of this system, sulforaphane [(\pm)-1-isothiocyanato-4-(methylsulfinyl)butane, 1] was recently isolated from Saga broccoli as the major phase 2 enzyme inducer present in organic solvent extracts of this vegetable.

Sulforaphane is of interest for three reasons: (i) it occurs naturally in a widely consumed vegetable; (ii) it is a very potent inducer of phase 2 enzymes; and (iii) it is a monofunctional inducer (4)—i.e., it elevates phase 2 detoxication enzymes without significantly changing the synthesis of cytochromes P-450 (5). These findings allowed the design and the systematic synthesis of a large number of structurally related isothiocyanates (6). It was found that the methylsulfinyl (CH_3SO) function of sulforaphane could be replaced by a methylcarbonyl (i.e., acetyl) group without significantly affecting inducer potency and that, in the most potent inducers, the isothiocyanate function and the acetyl group were separated by three or four carbons of an aliphatic or cyclo-aliphatic chain. Several isomeric norbornyl isothiocyanates substituted with acetyl groups were found to approach or equal the potency of sulforaphane as a phase 2 enzyme inducer (6). The merits of these norbornyl isothiocyanates [*exo*-2-acetyl-*exo*-6-isothiocyanatonorbornane, 2; *endo*-2-acetyl-*exo*-6-isothiocyanatonorbornane, 3; and *exo*-2-acetyl-*exo*-5-isothiocyanatonorbornane, 4] are that they can be more easily synthesized (from commercial 2-acetyl-5-norbornene) than sulforaphane and that they are probably more stable toward chemical and biological oxidation-reduction reactions.

We report here that sulforaphane and synthetic cyclic isothiocyanate analogues block mammary tumor development in Sprague-Dawley rats treated with 9,10-dimethyl-1,2-benzanthracene (DMBA) (7, 8). These findings identify a class of functionalized isothiocyanates as enzyme inducers that block carcinogenesis, and further strengthen the view that the search for phase 2 enzyme inducer activity from natural sources can successfully identify chemoprotectors against cancer (1-3). These results also encourage further efforts at rational design and laboratory synthesis of even more potent chemoprotectors.



MATERIALS AND METHODS

Animals and Mammary Tumor Development. Female Sprague-Dawley rats were obtained from Harlan-Sprague—

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Abbreviation: DMBA, 9,10-dimethyl-1,2-benzanthracene.
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Dawley Laboratories at 40 days of age. They were housed in plastic cages (four to five per cage) on Betachip hardwood laboratory bedding (Northeastern Product, Warrensburg, NY) and were fed unrestricted amounts of water and a pelleted AIN-76A diet containing no ethoxyquin (Teklad, Madison, WI). The temperature was 25°C, and 12-hr light/12-hr dark cycles were maintained. All animal experiments were in compliance with National Institutes of Health Guidelines (9) and were approved by the Animal Care and Use Committee of The Johns Hopkins University School of Hygiene and Public Health. The rats were assigned randomly to seven groups: a control group of 25 animals and six treatment groups of 20 animals each. The animals were weighed individually at weekly intervals. At age 47, 48, 49, 50, and 51 days, each animal received by gavage either 0.5 ml of Emulphor EL-620 (Rhône-Poulenc, Cranbury, NJ) alone or the specified doses (75, 100, or 150 μ mol daily) of sulforaphane (1) or compound 2, 3, or 4 in 0.5 ml of Emulphor EL-620. On day 50, 3 hr after administration of the vehicle or protector, all rats also received an intragastric instillation of 8.0 mg of DMBA (Sigma) dissolved in 1.0 ml of sesame oil. This dose of DMBA was selected to produce a substantial tumor incidence, but not one so high as to overwhelm a potential chemoprotective effect. The animals were examined once weekly for the appearance and location of palpable tumors. At age 202 days, i.e., 152 days after carcinogen administration, all animals were euthanized with ether and weighed. The tumors were separated from fat and connective tissue by dissection, weighed, and fixed in buffered 10% formalin. All tumors were identified microscopically by examination of stained sections.

Quantitative Assessment of Tumor Development. Of the 145 rats initially assigned to this experiment, 8 did not survive until we terminated the experiment when the rats were 202 days old. The deaths were distributed among the groups as follows: controls, 2 deaths with tumors; sulforaphane (150- μ mol dose), 1 death from gavage accident; sulforaphane (75- μ mol dose), no deaths; compound 2 (150- μ mol dose), 1 death with tumors; compound 2 (75- μ mol dose), 1 death with tumors; compound 3 (100 μ mol), 3 deaths, 2 with tumors, 1 without tumors; compound 4 (100 μ mol), no deaths.

Since tumor and body weights were not measured on the animals that died during the course of the experiment, we report separately the tumor incidence and multiplicity for all rats (two rats were excluded for reasons given in Table 1) and

for the 137 rats surviving to the termination of the experiment (Tables 1 and 2, respectively).

The mean body weights (\pm SEM) of the animal groups at the beginning of treatment (age 47 days) were between 116 \pm 1.7 and 126 \pm 1.9 g. The final weights at termination of the experiment are given in Table 2.

The development and characteristics of tumors in each group were assessed in four ways: (i) *incidence*, the fraction (percent) of animals that developed tumors; (ii) *multiplicity*, the total number of tumors divided by the total number of animals at risk; (iii) *total number and weight of tumors* removed from each animal at the termination of the experiment; and (iv) *latency of tumor development*. The proportions of tumor-free animals in the control and each treatment group were compared at the time of the weekly examinations of the animals (Fig. 1).

Statistical Analysis of Results. Differences in tumor incidence were evaluated by the Fisher exact test. Tumor multiplicity differences were analyzed by a Poisson distribution model and average rates were compared. The overall progression of tumor development was assessed by Kaplan-Meier analyses followed by logarithmic rank tests.

Chemical Syntheses. The synthetic methods and characterization of the compounds have been described (6). Multigram quantities of compounds 2-4 were prepared in one step from commercial 2-acetyl-5-norbornene (a mixture of *exo* and *endo* isomers) obtained from Aldrich.

RESULTS

Administration of sulforaphane or of the 2-acetyl-5-norbornyl isothiocyanates 2, 3, and 4 reduced the incidence, multiplicity, and weights and delayed the development of the mammary tumors evoked by a single dose of DMBA in female Sprague-Dawley rats (Tables 1 and 2; Fig. 1). There were clear-cut differences in the potencies of the chemoprotective compounds.

In the control group, not receiving any protector, the incidence of mammary tumors for all animals was 68.0% (Table 1). If the two animals that did not survive to the termination of the experiment (rat age, 202 days) were censored, the tumor incidence in the control group was very similar, 65.2% (Table 2). The corresponding tumor multiplicities (total number of tumors per number of animals at risk) were 1.56 (all animals) and 1.43 (survivors to termination),

Table 1. Protective effects of sulforaphane and norbornyl isothiocyanates 2, 3, and 4 on incidence and multiplicity of mammary tumors in DMBA-treated female Sprague-Dawley rats

Treatment group	No. of rats		Tumor incidence, % (% of control)	No. of tumors	
	In group	With tumors		Total	Multiplicity (% of control)
Control	25	17	68.0 (100)	39	1.56 (100)
Sulforaphane					
75 μ mol	20	7	35.0* (51.4)	9	0.45† (28.8)
150 μ mol	19‡	5	26.3* (38.7)	5	0.26† (16.7)
Compound 2					
75 μ mol	20	5	25.0* (36.8)	6	0.30† (19.2)
150 μ mol	20	5	25.0* (36.8)	7	0.35† (22.4)
Compound 3					
(100 μ mol)	19§	9	47.3 (69.6)	14	0.74† (47.4)
Compound 4					
(100 μ mol)	20	8	40.0 (58.8)	8	0.40† (25.6)

A total of 145 rats were entered into the experiment. Each received 8 mg of DMBA at age 50 days. There were initially 25 controls and 20 animals in each of the six treated groups. The above analysis is based on 143 animals (see below).

* $P < 0.05$ for differences from controls (Fisher exact test).

† $P \leq 0.01$ for differences from controls (Poisson distribution model).

‡One rat died immediately after gavage and is not included.

§One rat died without palpable tumors at age 167 days and is not included.

Table 2. Protective effects of sulforaphane and norbornyl isothiocyanates 2, 3, and 4 on incidence, multiplicity, and weights of mammary tumors in DMBA-treated female Sprague-Dawley rats: Analysis of survivors to termination of the experiment at rat age 202 days

Treatment group	No. of rats		Mean (\pm SEM) final body weight, g	Tumor incidence, % (% of control)*	Total no. of tumors in group*	Tumor multiplicity (% of control)*	Mean tumor weight, g (% of control)
	In group	With tumors					
Control	23 [†]	15	287 \pm 4.6	65.2 (100)	33	1.43 (100)	2.79 (100)
Sulforaphane							
75 μ mol	20	7	272 \pm 5.1	35.0 (53.8)	9	0.45 (31.4)	1.24 (44.4)
150 μ mol	19 [‡]	5	265 \pm 4.9	26.3 (40.3)	5	0.26 (18.2)	0.68 (24.4)
Compound 2							
75 μ mol	19 [§]	4	268 \pm 5.1	21.0 (32.2)	5	0.26 (18.2)	1.10 (39.4)
150 μ mol	19 [§]	4	274 \pm 7.0	21.0 (32.2)	5	0.26 (18.2)	1.12 (40.1)
Compound 3 (100 μ mol)	17 [¶]	7	276 \pm 4.4	41.2 (63.3)	12	0.71 (49.7)	2.49 (89.2)
Compound 4 (100 μ mol)	20	8	286 \pm 4.8	40.0 (61.3)	8	0.40 (27.9)	1.98 (71.0)

*The results shown in these columns differ from those in Table 1 because only the 137 rats that survived to termination of experiment are analyzed.

[†]Two animals died with tumors before termination of the experiment and are not included.

[‡]One rat died immediately following gavage and is not included.

[§]One rat in each group died with tumors before termination of experiment and is not included.

[¶]Three rats died (two with tumors) before termination of the experiment and are not included.

respectively. The mean weight of the control tumors at termination of experiment was 2.79 g (Table 2).

Sulforaphane administered in five doses of either 75 or 150 μ mol blocked tumor development in a dose-dependent manner. At the higher dose of sulforaphane, the tumor incidence and multiplicity for all animals in the group were reduced to 38.7% and 16.7% of control values, respectively (Table 1). The magnitude of this protective effect was almost identical (40.3% and 18.2%, respectively) when only those animals surviving to the termination of the experiment were analyzed (Table 2). Sulforaphane also reduced the tumor weights to 44.4% and 24.4% of controls at the lower and higher doses, respectively (Table 2). Treatment with sulforaphane significantly delayed the development of tumors during the course of the experiment in comparison to the control group ($P =$

0.016 and 0.002 at low and high doses of sulforaphane, respectively) (Fig. 1).

Norbornyl isothiocyanate 2 was an equally potent chemoprotector at the 75- and 150- μ mol doses, irrespective of whether incidence, multiplicity, tumor weight, or latency of tumor development was considered (Tables 1 and 2; Fig. 1). With these doses the protective effect appears to have attained a plateau. Since 75- μ mol doses of compound 2 reduced tumor incidence and multiplicity even more markedly than the same dose of sulforaphane, it is possible that 2 may be a more potent chemoprotector than sulforaphane.

Compounds 3 and 4, both tested at five doses of 100 μ mol, also blocked tumor formation, but these effects did not reach statistical significance for some indicators of protection (Tables 1 and 2). Thus, compound 3 was clearly the least potent

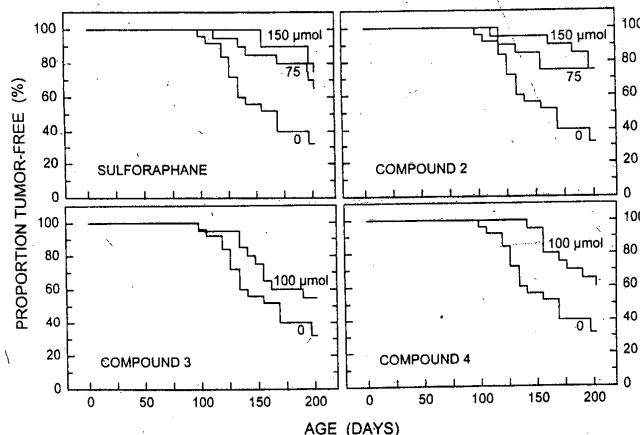


Fig. 1. Effect of treatment with sulforaphane (1) and norbornyl isothiocyanates (2-4) on mammary tumor development in female Sprague-Dawley rats that received 8 mg of DMBA at age 50 days. The proportion of tumor-free animals is shown at weekly intervals. The progression of tumor development in the control animals that received no protector (designated 0) is repeated in each panel. Sulforaphane: 75 μ mol ($P = 0.016$); 150 μ mol ($P = 0.0022$). Compound 2: 75 μ mol ($P = 0.023$); 150 μ mol ($P = 0.0074$). Compound 3: 100 μ mol ($P = 0.13$). Compound 4: 100 μ mol ($P = 0.023$). Kaplan-Meier incidence curves were analyzed by the logarithmic rank test. The P values refer to comparisons of differences in rate of tumor development in treated and control groups. All differences between treated and control groups are significant except for compound 3. The numbers of animals analyzed in each group are shown in Table 1.

protector. Although 3 produced some reduction in tumor incidence and multiplicity in comparison to controls, there was barely any reduction in tumor weights or significant prolongation in latency of tumor development. Compound 4 was apparently more potent than 3 but less potent than sulforaphane or compound 2 (Tables 1 and 2).

DISCUSSION

Sulforaphane, a naturally occurring isothiocyanate, and several structurally related synthetic 2-acetylnorbornyl isothiocyanates were tested for anticarcinogenic activity in this study, because they had previously been shown to be potent monofunctional inducers of phase 2 enzymes in cultured cells and in mouse tissues *in vivo* (5, 6). Since monofunctional induction has been proposed as a predictor of chemoprotective activity (7) and several structurally unrelated organic isothiocyanates have been shown to block chemical carcinogenesis and to induce Phase 2 enzymes (10, 11), we examined synthetic sulforaphane and the 2-acetylnorbornyl isothiocyanates 2–4 for chemoprotective activity. The present experiments demonstrate that all of these compounds, when administered around the time of carcinogen exposure, reduced—to varied degrees—the incidence, the multiplicity, and the weight of mammary tumors that developed in female Sprague–Dawley rats treated with DMBA. These agents also delayed tumor development. These observations further bolster the validity of the prediction that chemical compounds that induce phase 2 enzymes are promising candidates for achieving chemoprotection.

Sulforaphane was isolated as the principal and very potent phase 2 enzyme inducer from one variety of broccoli (5, 11). Our findings raise the issue to what extent sulforaphane and the many related inducers that are abundant in plants consumed by humans contribute to the chemoprotective activity of vegetables in man (12). There is insufficient information at present to draw conclusions on this matter.

Although our experiments do not provide a rigorous basis for comparing the relative potencies of sulforaphane (1) and compounds 2–4 as chemoprotectors, we can obtain some estimate of these potencies and relate them to the phase 2 enzyme inducer potencies (as measured by the concentrations required to double quinone reductase activities in murine hepatoma cells—i.e., the so-called CD values) (5, 6). These CD values are as follows: 0.2 μ M (sulforaphane); 0.3 μ M (compound 2); 0.8 μ M (compound 3); and 0.4 μ M (compound 4). A similar order of potency is reflected in the protective potencies of these compounds in blocking DMBA-induced mammary tumors. Compound 3 is the least potent protector. Sulforaphane and compound 2 are approximately equipotent, and 2 may be even more potent than sulforaphane. Sulforaphane and 2 are more potent than 3 or 4. It is therefore gratifying that measurements of inducer potencies in our cell culture assay not only correctly predicted anticarcinogenic activity but also provided a reasonable index of potency.

The reasons for the apparent differences in potencies of the compounds tested are not clear. The possibly higher potency of 2 in comparison to sulforaphane might be attributed to the

fact that the isothiocyanate group of 2 is secondary whereas that of sulforaphane is primary. Consequently, the former is likely to be less reactive and might therefore resist metabolic disposal or other promiscuous intracellular reactions with nucleophiles to which all isothiocyanates are susceptible. The differences in potencies of 2, 3, and 4 are more difficult to explain. In compounds 2 and 4 the functional groups are *exo*, whereas in compound 3 the 2-acetyl group is *endo* and, therefore, more protected. One aspect of this structure–activity relation is noteworthy: the nearly equivalent effects of methylsulfinyl ($\text{CH}_3\text{SO}-$) and methylcarbonyl ($\text{CH}_3\text{CO}-$) functions on both inducer and chemoprotective potencies of these agents.

The mechanisms of the chemoprotective actions of these compounds are not fully understood. Although isothiocyanates induce protective phase 2 enzymes, and the functionalized isothiocyanates used in these experiments are especially potent in this regard, it is becoming increasingly clear that some isothiocyanates also block activation of carcinogens by inhibiting phase 1 enzymes (10, 13). Whether sulforaphane and the 2-acetylnorbornyl isothiocyanates inhibit carcinogen activation is not known. Clearly, agents that are monofunctional inducers of phase 2 enzymes and block carcinogen activation by inhibiting phase 1 enzymes are likely to be ideal chemoprotectors.

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Regulatory Mechanisms of Monofunctional and Bifunctional Anticarcinogenic Enzyme Inducers in Murine Liver¹

Hans J. Prochaska² and Paul Talalay³

Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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ABSTRACT

Anticarcinogenic enzyme inducers are of two types: (a) bifunctional inducers [2,3,7,8-tetrachlorodibenzo-*p*-dioxin, polycyclic aromatics, azo dyes, β -naphthoflavone] that elevate both Phase II enzymes [e.g., glutathione *S*-transferases, UDP-glucuronosyltransferases, and NAD(P)H:(quinone-acceptor) oxidoreductase] and certain Phase I enzymes [e.g., aryl hydrocarbon hydroxylase (AHH)]; and (b) monofunctional inducers [e.g., diphenols, thiocarbamates, 1,2-dithiol-3-thiones, isothiocyanates] that elevate primarily Phase II enzymes without significantly affecting AHH. Since Phase I enzymes such as AHH may activate precarcinogens to ultimate carcinogens whereas Phase II enzyme induction suffices to achieve chemoprotection, an understanding of the molecular mechanisms that regulate these enzymes is critical for devising methods for chemoprotection. We report a systematic analysis of the inductions of aryl hydrocarbon hydroxylase (AHH) and NAD(P)H:quinone reductase (QR) by seven monofunctional and eight bifunctional inducers, singly or in combination, in a murine hepatoma cell line (Hepa 1c1c7) and two mutants defective in either *Ah* (Aryl hydrocarbon) receptor function (BP^c1) or in AHH expression (c1). We have also examined such inductions in genetically defined mouse strains with high affinity (CS7BL/6J) and low affinity (DBA/2J) *Ah* receptors. The combination of our earlier model for the induction of Phase I and Phase II enzymes (H. J. Prochaska, M. J. De Long, and P. Talalay, Proc. Natl. Acad. Sci. USA, 82: 8232, 1985) with mechanism(s) for autoregulation of AHH (O. Hankinson, R. D. Anderson, B. W. Birren, F. Sander, M. Negishi, and D. W. Nebert, J. Biol. Chem., 260: 1790, 1985) is compatible with our results. Thus, induction of QR by monofunctional inducers does not depend on a competent *Ah* receptor or AHH activity and appears to involve an electrophilic chemical signal. In contrast, bifunctional inducers require competent *Ah* receptors to induce both AHH and QR, although the latter process appears to be regulated by more than one mechanism. It is our view that bifunctional inducers bind to the *Ah* receptor thereby enhancing transcription of genes encoding both AHH and QR. Metabolizable bifunctional inducers are then converted by the induced AHH to products that resemble monofunctional inducers and are capable of generating the aforementioned chemical signal. The existence of mechanism(s) for AHH autoregulation that also affect Phase II enzyme expression would account for the high basal activities of QR in the AHH-defective mutant (c1).

INTRODUCTION

The protection of rodents against the toxic and neoplastic effects of chemical carcinogens can be achieved by a wide variety of seemingly unrelated chemical agents, including polycyclic aromatic hydrocarbons, azo dyes, flavonoids, phenolic antioxidants, isothiocyanates, diterpenes, indoles, unsaturated lactones, 1,2-dithiol-3-thiones, and thiocarbamates (1-4). Although a single mechanism cannot account for all forms of chemoprotection, it is clear that the induction of electrophile-

processing Phase II enzymes⁴ (e.g., glutathione *S*-transferases, UDP-glucuronosyltransferases, and quinone reductase) is a major protective mechanism (3, 4, 6-11). Indeed, the induction of these enzymes is the single common biochemical effect shared by the aforementioned compounds, and monitoring of Phase II enzyme induction has permitted the isolation and identification of new anticarcinogens (1, 3, 4, 6-10).

Although all of the chemoprotectors described above are inducers of Phase II enzymes, large differences exist among these agents in their capacity to induce certain Phase I enzymes (3, 4). Thus, large planar aromatics such as polycyclic aromatic hydrocarbons, flavonoids, TCDD,⁵ and azo dyes enhance selected Phase I activities such as aryl hydrocarbon hydroxylase in rodents and in cultured murine hepatocytes (10, 12-18), yet minor and variable inductions of Phase I enzymes are evoked by the remaining classes of compounds (3, 4). Thus, these anticarcinogens can be segregated into bifunctional inducers that induce both AHH and Phase II enzymes and monofunctional inducers that selectively induce Phase II enzymes only (3, 4, 6, 9). These families of inducers have been designated as type A and type B by Wattenberg (3). Since Phase I enzyme induction is an important mechanism for the activation of many carcinogens to ultimate electrophiles (10, 19), and hence counteracts chemoprotection, whereas Phase II enzyme induction results in chemoprotection, an understanding of the mechanisms underlying these inductions is of critical importance for devising appropriate strategies for chemoprotection.

The molecular mechanism whereby bifunctional inducers (large planar aromatics) elevate AHH and related activities appears to be well established. These compounds bind avidly to the protein product of the *Ah* (Aryl hydrocarbon) locus, and ligand-bound receptors bind to enhancer regions of selected cytochrome P-450 genes, resulting in elevation of AHH activity and increased metabolism of aromatic hydrocarbons (12, 16, 18, 20-24). Because the induction of Phase II enzymes by planar aromatics generally occurs only in mouse strains and cultured hepatocytes with a functional *Ah* locus, the regulation of these enzymes has been assumed to occur through the same mechanism as that regulating AHH (25-27). Nevertheless, experiments attempting to demonstrate the direct participation of the *Ah* locus in the regulation of Phase II enzymes have not been convincing (28-30).

In contradistinction, monofunctional inducers have little apparent structural similarity. Their mechanism does not appear

⁴ The enzymes involved in the metabolism of xenobiotics have been classified into two broad categories (5). Phase I enzymes (which include the cytochromes P-450) functionalize compounds by oxidation, reduction, or hydrolysis, whereas Phase II enzymes carry out the conjugations of functionalized compounds with endogenous ligands (e.g., glucuronic acid, glutathione, amino acids, and sulfate). Although quinone reductase does not promote a synthetic function, it may be classified as a Phase II enzyme since it does not introduce new functional groups, is often induced coordinately with conjugation enzymes, and protects cells against the toxicities of quinones (6).

⁵ Abbreviations and trivial names used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; QR, NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2) also known as DT-diaphorase or menadione reductase; AHH, aryl hydrocarbon hydroxylase (EC 1.14.14.1); *Ah*, Aryl hydrocarbon, the locus responsible for the induction by planar aromatics of aryl hydrocarbon hydroxylase (cytochrome P-450); Sudan III, 1-(4-phenylazophenylazo)-2-naphthol; oltipraz, 5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione.

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³ To whom requests for reprints should be addressed.

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MONOFUNCTIONAL AND BIFUNCTIONAL ENZYME INDUCERS

to involve a conventional receptor and does not require a functional *Ah* receptor (31, 32). Indeed, data presented previously (33) indicate that the inductive capacity of a wide range of phenolic antioxidants and phenylenediamines is critically dependent on the chemical properties of the inducers rather than their morphological features. Thus, in a murine hepatoma cell culture, a variety of hydroquinones (1,4-diphenols) and catechols (1,2-diphenols) are inducers of QR activity, whereas resorcinols (1,3-diphenols) are completely inactive. We have suggested that the observed inductions are the result of susceptibility to oxidations since catechols and hydroquinones have similar chemical properties in that they can undergo reversible one- or two-electron oxidations to the corresponding semiquinones or quinones, respectively. In contrast, resorcinols cannot undergo such facile oxidations. Hence, appropriate chemical reactivity rather than precise structure appears to be important for enzyme induction by diphenols. We have suggested that other classes of monofunctional inducers must possess electrophilic centers such as α,β -unsaturated carbonyl functions in order to exert inductive activity (34). Furthermore, we have proposed that the linkage of Phase II enzyme induction by bifunctional planar aromatics to *Ah* locus function may involve, at least in part, a metabolic cascade. Bifunctional inducers bind to the *Ah* receptor and thereby specify the enhanced synthesis of cytochrome P₁-450, which in turn converts bifunctional planar aromatics into species (e.g., 1,2- or 1,4-diphenols, diamines, aminophenols) that behave like monofunctional inducers (see Fig. 3).

Because of uncertainty over the relation between the regulation of the induction of these enzymes, we have undertaken a systematic analysis of this relationship with a variety of structurally dissimilar Phase II enzyme inducers in cultured cells and genetically defined mice. The induction of AHH activity, which is a measure of cytochrome P₁-450 levels, was used as an index of the induction of Phase I enzymes under the control of the *Ah* locus (12). QR activities were used as markers for Phase II enzyme induction. The majority of the experiments were done with the Hepa 1c1c7 murine hepatoma cell line in which the induction of both of these types of enzymes has been demonstrated (16-18, 20, 22-24, 31-33, 35-38). Induction experiments have also been carried out in two mutants of the Hepa 1c1c7 cell line that are defective in the *Ah* receptor or in AHH expression, as well as in mouse strains with high (C57BL/6J) and low (DBA/2J) affinity *Ah* receptors (12). These results have enabled the construction of a model for the mechanisms of induction of Phase I and Phase II enzymes.

MATERIALS AND METHODS

Materials. Flavin adenine dinucleotide, menadione, 2,6-dichlorodiphenol, bovine serum albumin, Tris base, Tween 20, 1-(2-pyridylazo)-2-naphthol, and 1-(2-thiazolylazo)-2-naphthol were obtained from Sigma (St. Louis, MO); 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was from IIT Research Institute (Chicago, IL); NADH was from Pharmacia P-L Biochemicals (Piscataway, NJ); 3-hydroxybenzo(a)pyrene was from the Chemical Carcinogen Reference Standard Repository (National Cancer Institute, Bethesda, MD); 75-cm² culture plates were from Falcon (Becton Dickinson Labware, Oxnard, CA); α -minimal essential medium and fetal calf serum were from GIBCO (Grand Island, NY); dimethyl sulfoxide, hexane, acetonitrile, and ethyl acetate were from Burdick and Jackson (Muskegon, MI); Emulphor EL-620P was from GAF (Linden, NJ); sesame oil was from Fisher (Fair Lawn, NJ). Other inducing agents were obtained and prepared as described previously (31, 33, 37). Hepa 1c1c7 cells and their mutants were gifts of J. P. Whitlock, Jr., Stanford University, and O. Hankinson, University of California, Los Angeles.

Treatment of Hepa 1c1c7 Cells and Assay of Enzymatic Activities. Wild-type and mutant Hepa 1c1c7 cells were plated, grown, and induced as described (31-33, 37). After 24 h of exposure to inducing agents, the cells were washed with ice-cold 0.15 M KCl-10 mM potassium phosphate (pH 7.4), removed from the plates by scraping, and sonically disrupted for 5 s (Branson Sonifier Cell Disruptor 200). Two 200- μ l aliquots of the resulting 1.0- to 1.5-ml suspensions were assayed for AHH activity as described by Nebert (39). The remaining samples were centrifuged at 5000 \times g for 20 min and assayed for quinone reductase activity by measuring the rate of oxidation of NADH (200 μ M) by menadione (50 μ M) at 340 nm in the assay system described by Prochaska and Talalay (40). Protein concentrations were determined by the method of Bradford (41).

Treatment of Animals. Female C57BL/6J and DBA/2J mice (The Jackson Laboratory, Bar Harbor, ME), 5 weeks old, were housed in hanging stainless steel cages (5 mice/cage) without bedding at 24-25°C with light-dark cycles of 12 h each. The mice were fed powdered 5001 Purina laboratory chow (Ralston-Purina, St. Louis, MO). After the mice were allowed to acclimatize to their new environment for 2 weeks, they were either: (a) fed by gavage one of the following test compounds: 75 μ mol of *tert*-butylhydroquinone, 35 μ mol of 3,5-di-*tert*-butylcatechol, or 35 μ mol of 4,6-di-*tert*-butylresorcinol, in 0.1 ml of Emulphor; or (b) given i.p. injections of test compound (5 μ mol of β -naphthoflavone, Sudan III, or 1-(2-thiazolylazo)-2-naphthol) in 0.2 ml of sesame oil. Both types of treatments were given daily for 5 days. Control groups received the respective vehicles only by the same routes. Mice were killed by cervical dislocation 24 h after the last dose and the livers were excised, perfused with 0.15 M KCl-2 mM EDTA (pH 7.4), frozen immediately in liquid nitrogen, and stored at -80°C until used.

Preparation of Mouse Liver Subcellular Fractions and Assay of Their Enzymatic Activities. Portions (500 mg) from each liver were homogenized in 1.5 ml of 0.15 M KCl, 10 mM potassium phosphate, and 0.5 mM EDTA, pH 7.4 ("homogenization buffer"). After centrifugation at 10,000 \times g for 30 min, the postmitochondrial supernatant fractions were centrifuged at 90,000 \times g for 75 min. The resulting cytosols were collected and assayed for: (a) QR by following the reduction of 2,6-dichlorodiphenol (40 μ M) by NADH (200 μ M) at 600 nm (40); (b) glutathione S-transferase with glutathione and 1-chloro-2,4-dinitrobenzene as substrates according to the procedure of Habig *et al.* (42); and (c) protein content by the method of Bradford (41). Microsomal pellets were suspended in 5 ml of homogenization buffer and centrifuged at 90,000 \times g for 90 min, resuspended in 0.5 ml of homogenization buffer, frozen in liquid nitrogen, and stored at -80°C. The microsomal fractions were subsequently assayed for: (a) AHH activity with benzo(a)pyrene and NADPH as substrates according to the method of Nebert (39); (b) P-450 levels according to the method of Omura and Sato (43); and (c) protein content according to the method of Bradford (41).

Statistical Treatment of Results. The results in the figures and Tables 1-3 are displayed as the ratios of the specific activities (or levels) of treated samples to those of controls. The standard error for each ratio was divided by the appropriate control value. The means \pm SE for the control groups are given in Tables 1 and 3 and in the appropriate figure legends. All results shown were obtained from at least duplicate measurements of four individual animals or four culture plates per treatment group.

RESULTS

In the studies described below we measured the specific activities of cytosolic QR and AHH in Hepa 1c1c7 cells and two mutants with defective AHH expression, as well as in livers of two inbred mouse strains, one of which (C57BL/6J) is responsive to induction of AHH by polycyclic hydrocarbons, while the other (DBA/2J) is unresponsive (12). To analyze the mechanisms of regulation of the induction of these enzymes by various chemoprotective agents we examined: (a) the relation of structure of the agents to their ability to induce the two types of activities; (b) dose-response relationships; and (c) the effects

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of combinations of maximally responsive concentrations of various types of inducers.

Induction of Quinone Reductase and Aryl Hydrocarbon Hydroxylase by Two Classes of Chemoprotectors. Fig. 1 provides a direct comparison of the induction in Hepa 1c1c7 murine hepatoma cells of QR (top) and AHH (bottom) by 15 compounds that are known to be inducers of these enzymes in rodent tissues (6). All of the compounds are recognized protectors against the neoplastic or toxic effects of chemical carcinogens (1-4, 6-9, 44, 45) under appropriate experimental conditions, although several are also carcinogens. In this experiment the hepatoma cells were grown to near confluence and then exposed for 24 h to concentrations of the inducers (10 nM to 30 μ M) selected to produce moderate to maximal inductions without causing obvious cytotoxicity. The results provide a striking and nearly absolute distinction between two types of inducers: (a) bifunctional inducers which are exemplified by polycyclic aromatics, 8-naphthoflavone, azo dyes, and TCDD; and (b) monofunctional inducers which include *tert*-butylhydroquinone, 3,5-di-*tert*-butylcatechol, bisethylxanthogen, 1,2-dithiol-3-thione, oltipraz, and benzylisothiocyanate. Whereas bifunctional inducers elevated both QR (2 to 4 times control levels) and AHH (10 to 30 times control levels), monofunctional inducers had no significant effect on AHH while inducing QR to values similar to those observed with bifunctional inducers.

One other important observation (Fig. 1) was that under identical conditions, the relative induction of the two enzyme activities by different bifunctional inducers varied considerably. For instance, at equimolar concentrations (2 μ M), benzo-

(a)pyrene and 3-methylcholanthrene were more effective than was Sudan III in inducing QR, whereas Sudan III produced a much greater induction of AHH than did the two polycyclic hydrocarbons. Furthermore, 1-(2-pyridylazo)-2-naphthol behaved anomalously from the other azo dyes in that it was a relatively poor inducer of AHH (about 2-fold) while raising the level of QR 3.5-fold. Since the ratios of induction of QR to AHH vary greatly from 0.0775 for Sudan III to 1.58 for 1-(2-pyridylazo)-2-naphthol, it seems evident that bifunctional inducers regulate QR and AHH by different mechanisms. Further evidence for several mechanism of induction will be developed below on the basis of dose-response curves, results obtained in mice *in vivo*, and the simultaneous use of saturating doses of both types of inducers.

Dose-Response Relationships in the Induction of Quinone Reductase and Aryl Hydrocarbon Hydroxylase by Monofunctional and Bifunctional Inducers. Because the degree of induction (ratio of treated to control specific activities) of AHH and QR by various inducers was not constant (Fig. 1), we examined the dose responses of these enzymes to selected compounds in the murine hepatoma Hepa 1c1c7 cell line (Fig. 2). The dose-response curves of induction of QR and AHH by TCDD were similar, suggesting a common mechanism of induction. TCDD was clearly the most potent inducer of both activities. TCDD was also the most effective inducer of AHH, yet it was not the most effective inducer of QR. Furthermore, differences in the regulation of AHH and QR by bifunctional inducers are apparent since the dose responses to Sudan III, 1,1'-azobenzene, and 1-(2-pyridylazo)-2-naphthol for these enzymes are quite different. Lastly, 1,2-dithiol-3-thione was completely inactive as an inducer of AHH yet it induced QR about as effectively as did Sudan III or TCDD.

Induction of Quinone Reductase and Aryl Hydrocarbon Hydroxylase by Combinations of Saturating Concentrations of Monofunctional and Bifunctional Inducers. Fig. 2 shows that concentrations of TCDD higher than 200 pM and of 1,2-dithiol-3-thione greater than 10 μ M produced maximal inductions of QR in Hepa 1c1c7 cells. The exposure of these cells to saturating concentrations of both compounds simultaneously produced

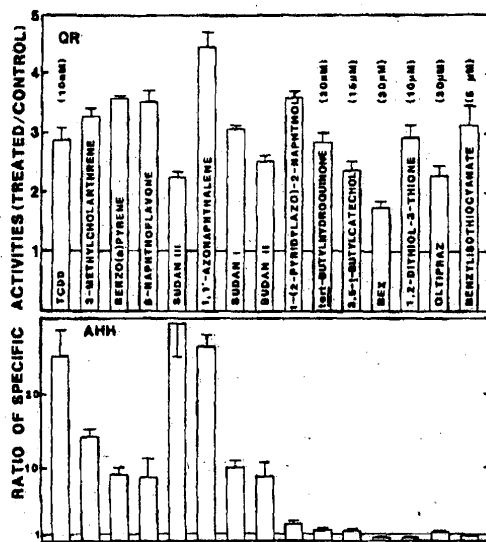


Fig. 1. Structure-activity study identifying monofunctional and bifunctional inducers. The induction profiles for QR and AHH in Hepa 1c1c7 murine hepatoma cells by 15 inducers of QR were determined and are expressed as specific activity ratios of treated to control cells. The concentrations of inducers were 2 μ M unless otherwise specified. Note that among polycyclic aromatics, the relationships between QR and AHH specific activities are not constant. The specific activities for QR and AHH in control cells were 297 ± 15 nmol/min/mg and 1.40 ± 0.14 pmol/min/mg, respectively. BEX, bisethylxanthogen; Sudan I, 1-(phenylazo)-2-naphthol; Sudan II, 1-(2,4-dimethylphenylazo)-2-naphthol.

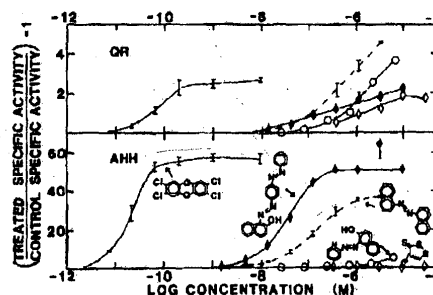


Fig. 2. Concentration dependence of induction of QR and AHH by TCDD, Sudan III, 1,1'-azobenzene, 1-(2-pyridylazo)-2-naphthol, and 1,2-dithiol-3-thione. The specific activities of QR and AHH in Hepa 1c1c7 murine hepatoma cells were determined as a function of concentrations and are expressed as ratios of treated to control cells (minus 1). TCDD is clearly the most potent inducer of both activities. TCDD is also the most effective inducer of AHH, yet it is not the most effective inducer of QR. 1,2-Dithiol-3-thione has no effect on AHH and the azo dyes tested show a variety of dose-concentration responses. These data suggest that multiple mechanisms for the induction of QR exist. The control values for QR and AHH were 349 ± 18 nmol/min/mg and 1.22 ± 0.08 pmol/min/mg, respectively.

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more than additive inductions of QR (Table 1). Thus 1–10 nM TCDD and 10–30 μ M 1,2-dithiol-3-thione elevated QR to 3.8 and 2.6 times control levels, respectively, whereas the combination produced more than an 8-fold induction. In contrast, the induction of AHH in Hepa 1c1c7 cells by saturating concentrations of TCDD (10 nM) was not affected by 30 μ M 1,2-dithiol-3-thione (Table 1) which by itself had a minimal effect on AHH activity (Figs. 1 and 2, Table 1).

Role of Ah Receptor Function in the Induction of Phase II Enzymes. Although the participation of the Ah receptor in the induction of Phase II enzymes by bifunctional inducers has been shown in inbred mice (25, 28, 29), such an association has not been clearly demonstrated with monofunctional inducers *in vivo*. We found that in livers of such inbred mouse strains (Table 2) monofunctional inducers acted independently of the Ah receptor since *tert*-butylhydroquinone and 3,5-di-*tert*-butylcatechol (but not 4,6-di-*tert*-butylresorcinol) induced glutathione S-transferase and QR in DBA/2J mice which have low affinity (defective) Ah receptors. The findings that 1,2- and 1,4-diphenols but not a 1,3-diphenol are inducers of QR in mice are

completely compatible with those reported with cell cultures (31–33). Furthermore, although planar aromatics such as Sudan III and β -naphthoflavone induced QR, glutathione S-transferase, and AHH activities in C57BL/6J but not in DBA/2J mice, the finding that 1-(2-thiazolylazo)-2-naphthol could induce Phase II enzymes in both strains of mice without greatly influencing AHH activity demonstrates that some bifunctional inducers may elevate Phase II enzymes in large part independently of the Ah receptor. Similar structure-activity relationships have been reported in rat liver and Hepa 1c1c7 cells (14, 31, 32).

To assess the role of the Ah receptor in the greater than additive induction phenomenon described above, the effects of combining saturating doses of monofunctional (1,2-dithiol-3-thione) with bifunctional (TCDD or β -naphthoflavone) inducers were examined in Hepa 1c1c7 cells as well as in its mutants defective in either a functional Ah receptor (BP^{c1}) (31, 32, 46) or the cytochrome P₄₅₀ gene (c1) (18, 38, 47). The BP^{c1} and c1 mutants have low and high basal specific activities of QR, respectively (Table 3; Refs. 31, 32, and 38). Neither mutant has detectable AHH activity. Table 3 shows that the combination of 1,2-dithiol-3-thione with either TCDD or β -naphthoflavone at saturating concentrations resulted in augmented induction of QR in Hepa 1c1c7 (wild-type) cells. Furthermore, an additive elevation of QR was observed when TCDD was combined with β -naphthoflavone. This increase in QR induction by addition of β -naphthoflavone to concentrations of TCDD that saturate all the Ah receptors argues that β -naphthoflavone induces QR via an alternate pathway. No combination of compounds gave AHH activities that were higher than those obtained with TCDD alone (not shown). In cells with defective Ah receptor function (BP^{c1}) or in mutants that produce a defective AHH gene transcript (c1), 1,2-dithiol-3-thione was the only compound tested that was capable of inducing QR effectively, although slight elevations of QR by bifunctional inducers were noted in the c1 mutant. The addition of bifunctional inducers to 1,2-dithiol-3-thione had no effect on the induction of QR in the BP^{c1} mutant although marginal increases were found with the c1 mutant. Hence monofunctional inducers act independently of the Ah receptor, whereas bifunctional inducers require

Table 1 Induction of quinone reductase and aryl hydrocarbon hydroxylase in Hepa 1c1c7 murine hepatoma cells by maximally inducing concentrations of TCDD and 1,2-dithiol-3-thione, singly and in combination

The results are expressed as ratios of specific activities of inducer-treated cells to controls.

Inducers	Concentration	Ratio of specific activities (treated/control)	
		Quinone reductase	Aryl hydrocarbon hydroxylase
TCDD	1 nM	3.66 \pm 0.09 ^a	30.4 \pm 1.8
	10 nM	3.92 \pm 0.03	36.8 \pm 5.6
1,2-Dithiol-3-thione	10 μ M	2.57 \pm 0.19	1.17 \pm 0.15
	30 μ M	2.67 \pm 0.10	1.29 \pm 0.05
TCDD and 1,2-dithiol-3-thione	10 nM	8.53 \pm 0.07 ^b	27.7 \pm 2.5
	30 μ M		

^a The standard error for each entry has been divided by the control value.

^b If the effects of TCDD and 1,2-dithiol-3-thione on QR were strictly additive a ratio of 3.59 would have been expected.

^c The mean control values for QR and AHH were (\pm SE) 230 \pm 5 nmol/min/mg and 0.959 \pm 0.069 pmol/min/mg, respectively.

Table 2 Induction patterns of hepatic quinone reductase, glutathione S-transferase, aryl hydrocarbon hydroxylase, and cytochrome P-450 levels in inbred Ah (aryl hydrocarbon) receptor-positive (C57BL/6J) and -negative (DBA/2J) mice

The results are expressed as ratios of specific activities (or levels) of treated to control livers. The means \pm SE were determined from four livers.

Inducer	Dose/day (μ mol)	Ratio of treated to control							
		C57BL/6J mice				DBA/2J mice			
		Quinone reductase	Glutathione S-transferase	Aryl hydrocarbon hydroxylase	Cytochrome P-450 levels	Quinone reductase	Glutathione S-transferase	Aryl hydrocarbon hydroxylase	Cytochrome P-450 levels
Sudan III	5	2.19 \pm 0.19	1.45 \pm 0.10	8.24 \pm 0.96	1.49 \pm 0.15	0.88 \pm 0.04	0.92 \pm 0.05	0.96 \pm 0.18	0.82 \pm 0.06
β -Naphthoflavone	5	3.59 \pm 0.26	2.15 \pm 0.05	6.70 \pm 0.44	1.56 \pm 0.17	1.74 \pm 0.05	1.29 \pm 0.05	0.94 \pm 0.26	0.93 \pm 0.11
1-(2-Thiazolylazo)-2-naphthol	5	4.17 \pm 0.18	2.56 \pm 0.11	2.20 \pm 0.41	0.85 \pm 0.10	1.94 \pm 0.16	1.86 \pm 0.15	1.05 \pm 0.14	0.87 \pm 0.05
3,5-di- <i>tert</i> -Butylcatechol	35	— ^a	—	—	—	3.83 \pm 0.32	4.74 \pm 0.34	0.69 \pm 0.07	0.83 \pm 0.14
4,6-di- <i>tert</i> -Butylresorcinol	35	— ^a	—	—	—	1.21 \pm 0.18	1.13 \pm 0.14	1.01 \pm 0.14	0.93 \pm 0.10
<i>tert</i> -Butylhydroquinone	75	2.12 \pm 0.08	2.15 \pm 0.13	1.23 \pm 0.11	1.22 \pm 0.14	2.37 \pm 0.13	2.65 \pm 0.24	0.98 \pm 0.18	0.73 \pm 0.13

Control values for mouse livers:

Mouse	Treatment	Glutathione S-transferase (nmol/min/mg)	Quinone reductase (nmol/min/mg)	Aryl hydrocarbon hydroxylase (pmol/min/mg)	Cytochrome P-450 levels (pmol/mg)
C57BL/6J	Sesame oil	2470 \pm 40 ^b	203 \pm 7	76.9 \pm 9.8	583 \pm 54
	Emulphor	2950 \pm 380	218 \pm 29	80.4 \pm 7.2	415 \pm 30
DBA/2J	Sesame oil	1890 \pm 100	160 \pm 16	84.6 \pm 4.5	437 \pm 45
	Emulphor	2190 \pm 240	144 \pm 14	74.9 \pm 10.2	491 \pm 35

^a Significant toxicity. Livers were not assayed.

^b Mean \pm SE of four livers.

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Table 3 Effect of combining bifunctional inducers with monofunctional inducers on the quinone reductase activity of Hepa 1c1c7 (wild-type), BPc1 (defective translocation of Ah receptor-ligand complex into nucleus), and c1 (defective cytochrome P₁-450 gene) cell lines

Cells were grown, induced, and assayed as described under "Materials and Methods." The treated/control ratios shown are the ranges for the means of two separate experiments. For each condition, three plates were assayed in one experiment and four in the other. Standard errors were less than 5% of the mean values. Note that aryl hydrocarbon hydroxylase was assayed and was found to be undetectable in BPc1 and c1 mutants, while the degree of induction of aryl hydrocarbon hydroxylase in Hepa 1c1c7 cells is consistent with the results shown in Figs. 1 and 2 (i.e., no compounds or combination of compounds was more effective than TCDD alone). Control quinone reductase specific activities (\pm SEM) in the two experiments, respectively, were: Hepa 1c1c7, 333 ± 5 and 223 ± 10 ; BPc1, 164 ± 3 and 113 ± 6 ; c1, 901 ± 20 and 937 ± 69 nmol/min/mg protein.

Inducers(s)	Concentration of Inducers	Ratio of quinone reductase specific activities (treated/control)		
		Hepa 1c1c7	BPc1	c1
1,2-Dithiol-3-thione	30 μ M	3.17-3.21	2.76-2.76	2.44-3.12
TCDD	10 nM	3.83-4.10	0.90-1.00	1.33-1.66
β -Naphthoflavone	5 μ M	5.13-6.07	0.87-0.98	1.20-1.36
1,2-Dithiol-3-thione + TCDD	30 μ M + 10 nM	9.55-9.59	2.60-2.66	3.21-4.53
1,2-Dithiol-3-thione + β -naphthoflavone	30 μ M + 5 μ M	7.08-7.63	2.53-2.71	2.42-4.71
TCDD + β -naphthoflavone	10 nM + 5 μ M	7.23-8.08	0.84-1.08	1.11-1.53

competent Ah receptors (also see Ref. 31 and 32). Furthermore, the role of AHH in the regulation of QR can be inferred from the results obtained with the c1 mutant since its genetic defect lies in the P₁-450 structural gene rather than the Ah receptor (47).

DISCUSSION

A model for the regulation of Phase II enzymes by monofunctional and bifunctional inducers proposed by us in 1985 (33) comprises three mechanisms (Fig. 3). In Mechanism A, monofunctional inducers activate the synthesis of Phase II enzyme by means of an electrophilic signal which operates independently of Ah receptors or the induction of AHH; in Mechanism B, complexes resulting from the combination of bifunctional inducers with Ah receptors bind to specific regions of nuclear DNA and thereby evoke enhanced transcription of both AHH and Phase II enzymes; and in Mechanism C, bi-

functional inducers susceptible to metabolism by AHH (induced by Mechanism B) are converted to electrophilic products that elevate Phase II enzymes by Mechanism A.

This model accounts for the observations obtained with Hepa 1c1c7 murine hepatoma cells. Thus, we have identified two families of Phase II enzyme inducers which are differentiated by their ability to induce AHH and their dependence (or independence) on Ah receptors for their mechanism of action. Nonmetabolizable bifunctional inducers such as TCDD (48) act via the Ah receptor directly (Mechanism B) to induce AHH and Phase II enzymes, while metabolizable inducers act also via Mechanisms C and A, whereby the induced AHH converts these compounds to metabolites resembling monofunctional inducers. This formulation would account for the similar dose-response curves of induction of QR and AHH by TCDD, the dissimilarity of these curves for metabolizable bifunctional inducers, and the disparity in the degrees of induction of AHH and QR evoked by various bifunctional inducers. Furthermore, the operation of two different yet interactive mechanisms (B and C/A) of induction on Phase II enzymes would be in agreement with the more than additive inductions observed by the use of combination of saturating concentrations of monofunctional and bifunctional inducers. The proposal that metabolizable bifunctional inducers act via more than one mechanism is also supported by the observation that saturating concentrations of TCDD and β -naphthoflavone produce virtually additive inductions of QR.

Most of the observations obtained with AHH-defective mutant cells are also consistent with the model shown in Fig. 3. Thus, monofunctional inducers elevate QR to the same degree in mutant as in wild-type cells, including the c1 mutant which has high basal QR activity. Bifunctional inducers are completely inactive in the BPc1 cell line since these cells have defective Ah receptors. This prevents bifunctional inducers from acting directly via Ah receptors (Mechanism B) as well as participating in a metabolic cascade (Mechanism C/A) since no AHH induction occurs. Surprisingly, bifunctional inducers were only weak inducers of QR in the c1 mutant. This was an unexpected result since this mutant has intact Ah receptors [the c1 mutant has its genetic defect in the cytochrome P₁-450 gene (47)]. Thus, bifunctional inducers should be able to induce QR by binding Ah receptors and directly activating genes coding for Phase II enzymes (Mechanism B).

In addition to the resistance of the c1 mutant to induction of QR by bifunctional inducers, there are other experimental findings with Hepa 1c1c7 cell mutants that cannot be accommodated by the above model. Hankinson *et al.* (18) observed that mutants with defects in their cytochrome P₁-450 gene had

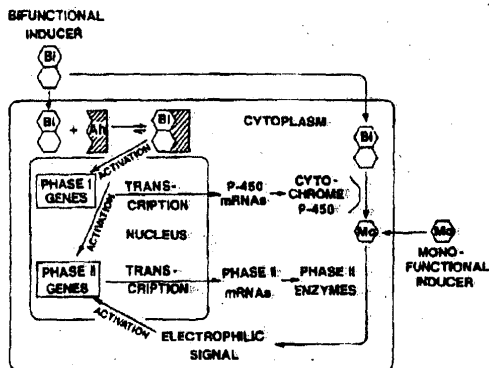


Fig. 3. Metabolic cascade model for the relation between the mechanism of action of monofunctional (Mo) and bifunctional (Bi) inducers of Phase I and Phase II enzymes (slightly modified from data of Prochaska *et al.* (33)). Monofunctional inducers enter the cell and generate the electrophilic signal that stimulates the induction of Phase II enzymes only. Bifunctional inducers require participation of the Ah receptor in two distinct mechanisms of induction. Bifunctional inducers enter the cell and bind to the Ah receptor, and the resultant complex activates gene transcription for both Phase I and Phase II enzymes. The resulting enhanced AHH activity converts metabolizable bifunctional inducers into compounds analogous in electrophilic properties to monofunctional inducers, which signal Phase II gene transcription. This model and mechanism(s) for AHH autoregulation that also affect Phase II enzyme expression (18, 38) can reconcile the experimental findings.

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high levels of P₄₅₀ mRNA which could not be elevated further by TCDD. These high P₄₅₀ mRNA levels were suppressed by coculture or fusion with wild-type cells. Two models were proposed to account for the regulation of cytochrome P₄₅₀ and the high basal activities of Phase II enzymes which are refractory to further elevations by TCDD (18, 38). In the first model, an endogenous ligand for the Ah receptor that is susceptible to inactivation by AHH is postulated. In cells without AHH, but which possess functional Ah receptors, the ligand accumulates, binds to Ah receptors, and activates the transcriptional activity of AHH and Phase II enzymes. In wild-type cells, the ligand cannot accumulate since it is rapidly inactivated by AHH. The second model proposes that prerepressors exist which are activated by AHH. The active repressors then decrease the transcriptional activity for AHH and Phase II enzymes. Hence, cells defective in AHH would not be able to convert prerepressors to active repressors that result in the high constitutive expression of AHH and Phase II enzymes.

We conclude that the model shown in Fig. 3 together with the postulated existence of mechanism(s) for the autoregulation of AHH that have similar effects on Phase II enzyme expression can account for the induction patterns of monofunctional and bifunctional inducers (alone or in combination), as well as the profiles of QR which Hepa 1c1c7 mutants exhibit.

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